Simplified Determination of Copper, Zinc, and Manganese in Plasma and Bile by Flameless Atomic Absorption Spectrometry

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CITATION:

SEKIYA, TSUKASA ...[et al]. Simplified Determination of Copper, Zinc, and Manganese in Plasma and Bile by Flameless Atomic Absorption Spectrometry. 日本外科宝函 1981, 50(6): 729-739

ISSUE DATE:

1981-11-01

URL:

http://hdl.handle.net/2433/208573

Arch Jpn Chir 50(6), 729～739, Nov., 1981

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Simplified Determination of Copper, Zinc and Manganese in Plasma and Bile by Flameless Atomic Absorption Spectrometry

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Received for Publication, Sept. 7, 1981.

Introduction

Copper, zinc and manganese are among the essential trace elements in humans, and they play important roles in clinical and pathological situations[28]. Although their functions are being clarified by many investigators, several aspects remain unknown because of the difficulty of assaying them in biological materials.

The methods in greatest use at this time are: neutron activation analysis[8,29], which requires a lengthy procedure and an expensive instrument, and atomic absorption spectrometry[5,10,12,13,14,16,17,18,21,25,26], which sometimes lacks sensitivity and requires relatively large samples.

Flameless atomic absorption spectrometry is very useful because it is highly sensitive, requires only a small sample and does not need a concomitant procedure. Measurements by this method of copper, zinc and manganese in some biological materials have recently been reported[2,3,4,6,7,9,11,15,19,20,22,23,24,27]. However, there has not yet been a wide application because of several technical difficulties. This report describes a rapid and simple method of determination of copper, zinc and manganese in plasma and in bile, which is the main excretory route of trace elements[28], with flameless atomic absorption spectrometry equipped with an autoinjector. The values obtained by the direct dilution method with pure water were compared with those obtained by the wet digestion method.

Experimental

Apparatus. An atomic absorption spectrometer with a deuterium arc background corrector and a signal integrator (Shimadzu AA 640-13 model, Kyoto, Japan) was used in conjunction with a graphite furnace atomizer (Shimadzu GFA-2 model), a peak catcher, a fast response

Key words: Flameless atomic absorption spectrometry, Trace element, Plasma, Bile, Copper, Zinc, Manganese.
索引語：フレームレス原子吸光分光光度計，微量元素，血漿，胆汁，銅，亜鉛，マンガン.
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recorder and an autoinjector instrument (Shimadzu AIU-1 model). The graphite furnace atomizer was purged with highly purified argon gas at a flow rate of 1.0 L/min. The graphite furnace atomizer was operated by setting the time and electric current values of three stages (drying, ashing and atomizing) and selecting gas three mode channels. Argon gas flows throughout the three stages in gas mode 1, during the drying and ashing stages in gas mode 2 and only during the atomizing stage in gas mode 3. The sensitivity is highest when gas mode 2 is chosen. Samples were injected into the center hole of the graphite tube by the autoinjector equipped with a 10 μl Eppendorf micropipette fitted with a disposable tip, moved by compressed air and rinsed with pure water. It is very easy to inject samples correctly with this system.

Reagents. Pure water was obtained by an infrared, nonboiling type distillation instrument (Daiken Quartz Glass, Tokyo, Japan). Zinc is the most ubiquitous concomitant, but water purified in this instrument contains very little zinc under 0.5 ng/L and no copper or manganese. This water was used throughout the study. Working standard solutions were prepared by diluting 1000 mg/L of stock solutions of copper, zinc and manganese (Kanto Chemical, Tokyo, Japan) with pure water. These were stored in borosilicate flasks at room temperature. The ion concentrations were determined weekly, and almost no change was found for one month. The working standard solutions contained 50 μg/L to 200 μg/L of copper and 5 μg/L to 20 μg/L of zinc and manganese. HNO_3, HCl and $HClO_4$ used for cleaning glassware and in the wet digestion method were all reagents for poisonous metal analysis (Kanto Chemical), and are the most highly purified reagents which contain no copper or manganese, but do contain about 3 μg/L of zinc.

Glassware cleaning. In order to minimize contamination, great care was taken throughout the analytical work. Glassware was rinsed many times with pure water and immersed in 4N-HCl for one week. Then it was rinsed repeatedly with concentrated HCl and pure water. The adequacy of the cleaning was checked by analysing the rinse water.

Collection and preparation of samples. Plasma was obtained by Venoject (Terumo, Tokyo, Japan) from postoperative patients or healthy adult volunteers, and bile samples were collected from choledochal tube inserted during surgery for cholelithiasis, dropping into polypropylene tubes. In collection of samples metals are frequently added as contaminants, as commented by VERSIECK J[30]. So we measured pure water which was injected by Venoject (green stopper), it contained only 15 μg/L of zinc and no copper or manganese. Choledochal tubes made of rubber, are not adequate since they contain a large amount of zinc, but siliconized tubes are zinc-free. Pure water which was collected after passage through siliconized tubes contained no metals. In the direct dilution method, samples were merely diluted with enough in polypropylene tubes to reach the detection range. In the wet digestion method, 5 ml of plasma or bile was completely dissolved with concentrated HNO_3 and $HClO_4$ on a hot plate for about four hours and then added to pure water in borosilicate flask to make up 50 ml, as described by BUCHET JP[4]. To eliminate the contamination error during the digestion procedure, an acid blank was always prepared in the same way.

Firstly, operating parameters were studied using working standard solutions. The optimal

conditions for copper, zinc and manganese were found to be different, so they were studied separately. In the case of copper and manganese, it was easy to obtain satisfactory reproducibility and linearity of standard calibration curves. For zinc, however, since absorbance signals were too labile in gas mode 2, gas mode 1 was chosen; although the sensitivity was lower, it was still high enough to measure samples. On starting the measurement of diluted samples, we encountered the problem of poor reproducibility although previous researchers have reported the direct determination of plasma zinc and manganese without pretreatment[9,15,23].

Results and Discussion

Operating parameters. It was found that the reproducibility was much more affected by various factors than when standard solutions were analyzed. Operating parameters for plasma and bile samples of copper, zinc and manganese were determined as follows with standard solutions used as reference (Figures 1 and 2). In the drying stage, if an electric current higher than 20 A was used, the samples spluttered and foamed in the graphite tube. Therefore, a low electric current (15 A) was used for a longer time (40 sec.) in all cases. In the atomizing stage, an electric current producing as high as possible an absorbance signal was selected for zinc and manganese. However, since the atomizing temperature is low for zinc, other substances might remain after the end of the measurement and interfere with the next determination. Therefore, as soon as the

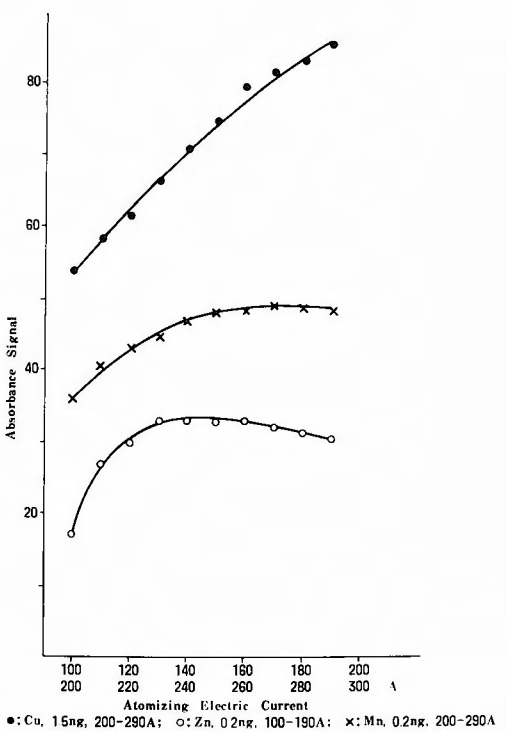

Fig. 1. Effect of atomizing electric current on the absorbance signal for copper, zinc and manganese

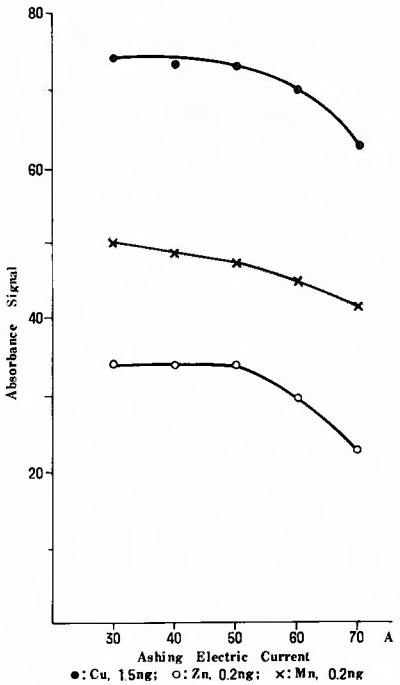

Fig. 2. Effect of ashing electric current on the absorbance signal for copper, zinc and manganese

atomizing stage ended, 300 A of electric current was produced by turning on the autocleaning switch to clean the graphite tube. With copper, the higher the electric current, the higher was the absorbance signal. We chose 250 A fearing the destruction of the graphite tubes. The ashing stage is the most important of the three stages, because organic substances in the samples make smoke in the atomizing stage with a low electric current, and some metals may evaporate out with a high one. Therefore optimal parameters must be chosen carefully. The volume of the sample to be injected was determined from the space it took up in the graphite tube and the absorbance signal. When it was less than 5 μl, the signal was too low and when it was more than 20 μl, the sample bulged out from the tube. So 10 μl was chosen. The samples must be injected at the same point without touching the tube because of the difference of temperature between the central and peripheral portions of the graphite tube[27,31]. For this purpose, the autoinjector is very helpful. To study operating parameters, it is useful to look through the hole of the graphite tube. All analyses were carried out under the conditions listed in Table 1. There were no differences among standard solutions, plasma and bile.

Reproducibility. The precision of the dilution method was tested by repeating 10 determinations each of the copper, zinc and manganese in the standard solutions, diluted plasma and bile, as shown in Figure 3. For plasma copper, the dilution ratio was 10 fold and for plasma zinc, it was 100 fold. In bile, the concentrations of the three metals differed markedly, so various dilution ratios were used appropriate for the detection range. The results, as summarized in Table 2, were satisfactory except for plasma manganese. The reproducibility of the three metals was

Table 1. Operating parameters for copper, zinc and manganese in plasma and bile

	Cu	Zn	Mn
Wavelength	324.7 nm	213.9 nm	279.5 nm
Slit setting	1.9 Å	1.9 Å	1.9 Å
Lamp current	2 mA	4 mA	4 mA
D_2 Lamp intensity	4	3	3
Mode	B.G.C.*	B.G.C.*	B.G.C.*
Signal integrater	3 sec,	3 sec,	3 sec,
Drying stage	15 A, 40 sec,	15 A, 40 sec,	15 A, 40 sec,
Ashing stage	50 A, 30 sec,	40 A, 30 sec,	50 A, 30 sec,
Atomizing stage	250 A, 5 sec,	140 A, 5 sec,	250 A, 5 sec,
Autocleaning switch	Off	On	Off
Gas mode	2	1	2
Argon gas flow	1 L/min.	1 L/min.	1 L/min.
Injected volume	10 μl	10 μl	10 μl

*B.G.C.=Background correction with a deuterium arc background corrector

compared. Copper was the most consistent, its C.V. (coefficient of variation) being under 4.9%. Both plasma and bile samples were less consistent than the standard solutions, presumably because of the difference of viscosity of the materices, because a little of the sample of plasm or bile sometimes remained in the tip of the Eppendorf micropipette instead of being completely pushed into the graphite tube. The concentration of manganese in plasma is much lower than that of the other two metals[9,20], so it is necessary to use dilution ratios lower than 10 fold or to increase the scale expansion of the recorder. However, even though we tried various parameters, we could not obtain satisfactory results. On the other hand, the C.V of four plasma samples after the wet digestion procedure was 5.7±2.6%.

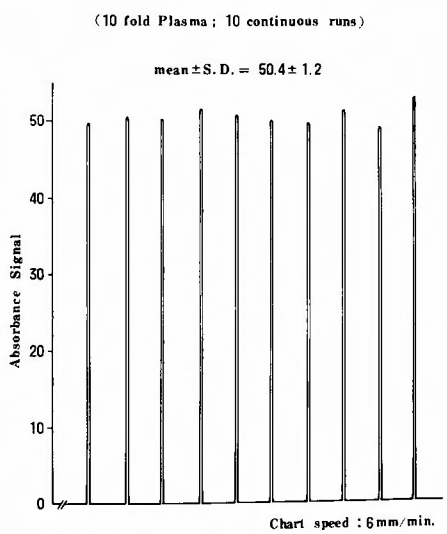

Fig. 3. Reproducibility test for copper in plasma

Table 2. Reproducibility of various samples with the dilution method (10 continuous runs)

Metals	Samples	Absorbance Signal*	C.V. (%)**
Cu	50 μg/L working standard solution	23.3±0.39***	1.7
	100 μg/L working standard solution	48.4±0.93	1.9
	10 fold diluted plasma	50.4±1.20	2.4
	direct bile	15.7±0.33	2.1
	2 fold diluted bile	11.0±0.54	4.9
	10 fold diluted bile	12.5±0.48	3.8
Zn	10 μg/L working standard solution	16.8±0.49	2.9
	20 μg/L working standard solution	30.4±0.73	2.4
	100 fold diluted plasma	14.5±0.85	5.9
	10 fold diluted bile	13.2±0.89	6.7
	20 fold diluted bile	15.5±1.02	6.6
Mn	10 μg/L working standard solution	23.3±0.58	2.5
	20 μg/L working standard solution	47.2±12.7	2.7
	10 fold diluted bile	26.9±1.42	5.3
	20 fold diluted bile	24.5±1.10	4.5

* Recorder scale expansion: ×1, ** Coefficient of variation
*** Mean±Standard deviation

Ion interference. In a study of the effect of Na, K, Ca and Cl on copper, zinc and manganese, calibration curves were prepared as in the standard additon method by adding 20 μl of standard solutions of the three metals to Sorbit Hartmann® solution (Nikken Chemical, Tokyo, Japan), which simulates the extracellular fluid of the human body (Table 3). This solution contains 6.2 μg/L of zinc contamination but no copper or manganese. The calibration curves with the use of the deuterium arc background corrector were linear and parallel to the standard calibration curves for each metal (Figure 4). Furthermore, calibration curves were prepared by solutions of 100 μg/dL of Fe and 2.0 mg/dL of Mg, and the same result. No interference was observed in assaying of biological fluid.

Recovery. In a further estimation of the effect of the matrices, recovery tests were carried out as follows: 2 ml of a diluted or nondiluted sample was mixed with 20 μl of a standard solution (copper: 5 μg/ml; zinc and manganese: 0.5 or 1 μg/ml). The final added concentration of was 50 μg/L, and of zinc and manganese 5 μg/L or 10 μg/L, which was adequate for the detection

Table 3. Composition of Sorbit Hartmann® solution

Na^+	130.3 mEq/L
K^+	4.0 mEq/L
Ca^{++}	2.7 mEq/L
Cl^-	109.4 mEq/L
Lactate	27.7 mEq/L
Sorbitol	5.0 w/v%

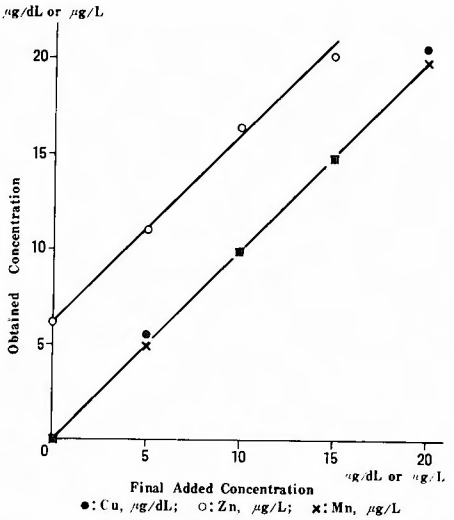

Fig. 4. Calibration curves of copper, zinc and manganese added to sorbit hartmann® solutions

range. Then the average of two signals (three signals if two signals differed by more than 10%) was employed. Table 4 shows the results. Plasma manganese was excluded, since the reproducibility was poor. The recovery of three metals in plasma and bile showed individual variations but averaged 95.7% to 103.5% and was satisfactory on the whole.

Comparison with the wet digestion method. The accuracy of the dilution method was tested by comparing it with the wet digestion method in determining the amounts of the three metals in four plasma and four bile samples with the same instrument under the same conditions. After the wet digestion procedure described above, an acid blank signal must be subtracted. In 20

Table 4. Recovery of copper, zinc and manganese added to plasma and bile

Metals	Samples	Number of samples	Amount added	Recovery (%)	Mean±S.D.*
Cu	10 fold diluted plasma	4	50 μg/L	88.0–106	101 ±9.0
	direct bile	1			
	4 fold diluted bile	2	50 μg/L	86.0–108	95.7±7.8
	10 fold diluted bile	4			
Zn	100 fold diluted plasma	4	5 μg/L	94.0–106	103.5±7.2
	100 fold diluted plasma	4	10 μg/L	95.0–109	102.3±6.1
	10 fold diluted bile	1			
	20 fold diluted bile	2	5 or 10 μg/L	88.9–109	99.8±6.3
	40 fold diluted bile	2			
Mn	2 fold diluted bile	2			
	10 fold diluted bile	2	5 or 10 μg/L	87.6–104	97.1±6.3
	40 fold diluted bile	2			

*S.D.=Standard deviation

Table 5. Comparison with wet digestion method

		Cu		Zn		Mn		
		x	y	x	y	x	y	(μg/dL)
Plasma	1.	71.3	71.6	85.0	90.0	1.9		
	2.	83.0	81.0	115	108	2.3		
	3.	73.8	73.8	88.0	79.0	1.8		
	4.	80.4	70.6	68.5	80.0	0.8		
Bile	a.	65.5	65.0	37.9	36.0	15.9	16.0	
	b.	73.4	78.3	10.2	9.8	47.3	45.8	
	c.	303.3	278.3	29.6	24.4	34.7	34.0	
	d.	54.2	53.0	225.6	217.9	18.3	16.6	

x; Wet digestion method y; Direct dilution method
($y=0.929x+3.00$, $r=0.997$, $n=20$)

cases, excluding plasma manganese, there was no significant difference in the results obtained by the two methods, as shown in Table 5. On regression analysis, $y=0.929x+3.00$ μg/dl, $r=0.997$, $n=20$, $p<0.001$, (y: direct dilution method; x: wet digestion method; r: correlation coefficient). The accuracy of this method was also verified by the results.

Clinical use. Plasma samples obtained from 13 "non fasting" healthy volunteers and 11 human bile samples collected at 9 : 00 A.M. from choledochal T tubes (siliconized) were assayed by this method. The mean values of copper and zinc in plasma, shown in Table 6, are lower than those noted in previous reports: copper, 94–106 μg/dl; zinc, 82–113 μg/dl[1,5,10,13,17,21,29]. We consider that the difference is probably due to the time of collection and the freedom from contamination[10,21]. This is the first time this instrument has been used to measure metals in bile and the concentrations of each metal showed a wide range (Table 7): copper, 46 μg/L to 840 μg/L, zinc 26 μg/L to 743 μg/L and manganese 20 μg/L to 583 μg/L. It is very interesting that there is more manganese in bile than in plasma.

Flameless atomic absorption spectrometry is an excellent technique, but it has a few defects, such as inferior reproducibility and a narrow detection range. We have expended much effort to improve the reproducibility, because further examination are impossible if reproducibility is poor. In this respect, it would be most important that the sample volume was correctly injected into the graphite tube. Concerning the matter, an autoinjector contributed the successful results by the dilution method. Then, as described by ROBBINS W.B.[22], it may be useful to drill and widen a hole of the tube.

One of our purposes is to establish a simple, rapid and especially suitable technique for large numbers of biological samples. Every reagent is contaminated with zinc, more or less, so con-

Table 6. Copper and zinc concentrations in plasma

N=13	Cu	Zn	μg/dL
Plasma	82.3±17.0	77.9±17.8	
	(Mean±S.D.)		

Table 7. Copper, zinc and manganese concentrations in human bile

		Cu	Zn	Mn	
Bile	1.	690	320	82	
	2.	236	182	38	
	3.	56	196	80	
	4.	62	26	20	
	5.	504	75	155	
	6.	420	51	21	
	7.	840	372	415	
	8.	162	743	583	
	9.	46	605	39	
	10.	542	177	25	
	11.	105	232	82	
Mean±S.D.		333±280	271±228	140±185	(μg/L)

comitant procedure increases opportunities for contamination. From this point of view, flameless atomic absorption spectrometry with the direct dilution method is quite useful compared to other methods, except for plasma manganese, for which a certain pretreatment may be necessary to increase the accuracy.

Acknowledgement

It is a pleasure to thank Associate Professor R.D. SOLOWAY, Gastrointestinal Section of the Department of Medicine, Hospital of the University of Pennsylvania, for valuable advice and encouragement. This work was in part supported by Grant- in-Aid No. 00448253 for Scientific Reseach from the Ministry of Education, Science and Culture 1981.

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和文抄録

フレームレス原子吸光分光光度計による血漿および胆汁中銅，亜鉛，マンガンの測定

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生体中微量元素は極めて濃度が低く，また広く生物環境中に存在するため常に試料汚染の危険性があり，その正確な測定は分析技術の進んだ今日においてもなお，非常に困難である．それが，生体中の微量元素の代謝に関する研究を妨たげている大きな要因である．生体試料の分析には，主としてフレーム原子吸光法や放射化分析法により行われているが，前者は，感度が低く，かなりの量の試料を必要とする欠点があり，また後者は，多大な装置，繁雑な前処理を要するため一般的ではない．

フレームレス原子吸光分光光度計は，従来の原子吸光分光光度計に比べ格段の高感度を有し，しかも極く微量の試料で測定可能という大きな特徴をもっている．しかし，その反面，あまりに高感度であるゆえに，試料の Contamination や装置の setting 条件等により大きな影響を受け，精度の点，特に再現性において他の分析機器に比べ劣り，生体材料への応用が普及しなかった．それゆえ，胆汁は，微量元素の重要な排泄経路であるにも拘らず，いまだその検討がなされないままになっている．

われわれは，D_2 ランプによるバックグラウンド補正装置を有する島津 AA 640-13 型原子吸光分光光度計，グラファイトファーネスアトマイザー (GFA 2 型)，シグナルピークキャッチャー (PCA-1) に，試料注入時の精度を高めるため自動注入器 (AIU-1) を装備し，血漿中，胆汁中銅，亜鉛，マンガンの測定法について検討した．

血漿は，金属含量の最も少い Venoject で，胆汁はゴム製Tチューブを避け，シリコン製Tチューブからポリプロピレン製チューブに落下させて採取し，contamination を最小限に抑えるとともに，赤外線非沸騰型蒸留装置で精製した純水を用い，希釈のみにて直接測定する方法を検討し，以下の結果を得た．

1) 再現性：10回連続注入による変動係数は，血漿中マンガンを除いて6.7%（胆汁中亜鉛）以下と満足できる成績であった．ただし，血漿中マンガンは，S/N 比が低く希釈倍率が低いため，粘稠な試料の注入を必要とし，再現性が劣っていた．

2) イオン干渉：他のイオンによる干渉は，通常生体中に含まれる程度の濃度では，バックグラウンド補正により，なんら影響はなかった．

3) 添加回収率：血漿および胆汁中における各元素の平均添加回収率は，95.7%から103.5%と良好であった．

4) 湿式灰化法との比較：血漿および胆汁，計20個の場合について検討したが，$r=0.997$ と高い相関性を認めた．

5) 臨床応用：血漿13試料中の銅および亜鉛，ヒト胆汁8試料上の銅，亜鉛およびマンガンをそれぞれ測定した結果，血漿中平均銅濃度は，82.3±17.0 μg/dl，亜鉛濃度は，77.9±17.8 μg/dl と諸家の報告よりやや低かったが，これは測定時の contamination を最小限に防げたことを示唆している．胆汁中銅濃度は，46～840 μg/L，亜鉛濃度 26～743 μg/L，マンガン濃度 20～583 μg/L と，個体差は極めて大きかった．

以上の成績から，血漿および胆汁中微量元素の測定法として本法は，迅速かつ簡便で多量の検体を処置することができ，甚だ有用であるといえよう．